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Identification of p130<sup>cas</sup> as a Substrate for the Cytosolic Protein Tyrosine Phosphatase PTP-PEST

ANDREW J. GARTON, ANDREW J. FLINT, AND NICHOLAS K. TONKS\*

Cold Spring Harbor Laboratory, Cold Spring Harbor,  
New York 11724-2208

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PTP-PEST is a ubiquitously expressed, cytosolic, mammalian protein tyrosine phosphatase (PTP) which exhibits high specific activity in vitro. We have investigated the substrate specificity of PTP-PEST by a novel substrate-trapping approach in combination with in vitro dephosphorylation experiments. We initially identified a prominent 130-kDa tyrosine-phosphorylated protein in pervanadate-treated HeLa cell lysates which was preferentially dephosphorylated by PTP-PEST in vitro. In order to identify this potential substrate, mutant (substrate-trapping) forms of PTP-PEST were generated which lack catalytic activity but retain the ability to bind substrates. These mutant proteins associated in stable complexes exclusively with the same 130-kDa protein, which was identified as p130<sup>cas</sup> by immunoblotting. This exclusive association was observed in lysates from several cell lines and in transfected COS cells, but was not observed with other members of the PTP family, strongly suggesting that p130<sup>cas</sup> represents a major physiologically relevant substrate for PTP-PEST. Our studies suggest potential roles for PTP-PEST in regulation of p130<sup>cas</sup> function. These functions include mitogen- and cell adhesion-induced signalling events and probable roles in transformation by various oncogenes. These results provide the first demonstration of a PTP having an inherently restricted substrate specificity in vitro and in vivo. The methods used to identify p130<sup>cas</sup> as a specific substrate for PTP-PEST are potentially applicable to any PTP and should therefore prove useful in determining the physiological substrates of other members of the PTP family.

The protein tyrosine phosphatase (PTP) family of enzymes consists of more than 75 structurally diverse proteins which have in common the highly conserved 250-amino-acid PTP catalytic domain, but which display considerable variation in their noncatalytic segments (5, 56). This structural diversity presumably reflects the diversity of physiological roles of individual PTP family members, which in certain cases have been demonstrated to have specific functions in growth, development, and differentiation (11, 25, 36, 39, 49). Although recent studies have also generated considerable knowledge regarding the structure, expression, and regulation of PTPs, the nature of the tyrosine-phosphorylated substrates through which the PTPs exert their effects remains to be determined. Studies with a limited number of synthetic phosphopeptide substrates have demonstrated some differences in substrate selectivity of different PTPs (7, 8) and have indicated preferences for certain amino acid residues at particular positions around the phosphorylated tyrosine residue (44, 63). This indicates that PTPs display a certain level of substrate selectivity in vitro, although the physiological relevance of the substrates used in these studies is unclear.

PTP-PEST is an 88-kDa cytosolic PTP (6, 9, 54, 58, 59) which is expressed ubiquitously in mammalian tissues (60) and which exhibits high specific activity when assayed in vitro with artificial tyrosine-phosphorylated substrates (15). We have previously demonstrated that PTP-PEST is subject to regulation via phosphorylation of Ser-39 in vitro and in vivo. This modification is catalyzed by both protein kinase C and protein

kinase A and results in reduced enzyme activity as a consequence of an increase in the  $K_m$  of the dephosphorylation reaction (15). It appears likely that further regulatory mechanisms exist for PTP-PEST, since this enzyme would be expected to exert a considerable negative influence on the tyrosine phosphorylation state of cytosolic substrates of tyrosine kinases. One possibility is that this influence could be limited by the substrate specificity of PTP-PEST, of which details are currently lacking.

The crystal structures of PTP1B alone (2) and in a complex with a phosphotyrosine-containing peptide (21) were recently determined. These structures indicated roles for the invariant residues of the PTP catalytic domain and suggested a mechanism for catalysis which is consistent with the available kinetic data and which may be applicable to PTPs in general (3). After binding of phosphotyrosine in the active site cleft, a thiol-phosphate intermediate is formed between the substrate and the active site cysteine residue (17). In addition, substrate binding induces movement of a loop that forms one side of the active site cleft (equivalent to amino acids 179 to 187 in PTP1B and residues 197 to 205 in PTP-PEST), resulting in a more closed structure around the active site. In PTP1B, this conformation is stabilized by hydrophobic interactions between the phosphotyrosine phenyl ring and the side chain of Phe-182. The movement of this loop brings the side chain of an invariant, catalytically essential aspartic acid residue (Asp-181, equivalent to Asp-199 in PTP-PEST) into position to act as a general acid in the catalytic mechanism (10, 64); by donating a proton to the substrate tyrosine residue, this aspartic acid residue destabilizes the transition-state intermediate, resulting in the release of the dephosphorylated peptide from the active site. The essential catalytic role of Asp-181 in PTP1B is supported by the kinetic properties of a mutant protein in which this residue is changed to Ala (13). The resultant enzyme

\* Corresponding author. Mailing address: Cold Spring Harbor Laboratory, 1 Bungtown Rd., Cold Spring Harbor, NY 11724. Phone: (516) 367-8846. Fax: (516) 367-6812. Electronic mail address: tonks@cshl.org.

displays a similar  $K_m$  but a greatly reduced  $V_{max}$  compared with the wild-type enzyme. These observations suggested that the D181A (D-181→A change) mutant enzyme could potentially yield an inactive enzyme which largely retains the ability to bind substrates; these properties would clearly be useful for trapping potential PTP substrates to facilitate their isolation and identification. We have utilized such a mutant form of PTP-PEST in these experiments.

We have used pervanadate-treated cells as an abundant source of tyrosine-phosphorylated proteins to investigate the substrate specificity of PTP-PEST. Using a combination of in vitro dephosphorylation and substrate-trapping experiments, we have found that PTP-PEST exclusively selects p130<sup>cas</sup> as a substrate from complex mixtures of 50 to 100 phosphotyrosine-containing proteins. Our results strongly suggest that the physiological role of PTP-PEST involves regulation of the phosphorylation state of p130<sup>cas</sup> and are the first direct demonstration of a PTP having an inherently restricted substrate specificity.

#### MATERIALS AND METHODS

**Generation, expression, and purification of mutant PTP proteins.** Point mutations within the catalytic domains of PTP-PEST (D199A and C231S) and PTP1B (D181A and C215S) were introduced by site-directed mutagenesis with the Muta-Gene in vitro mutagenesis kit (Bio-Rad, Richmond, Calif.). Regions containing the required point mutation were then exchanged with the wild-type sequences within appropriate expression vectors, and the replaced mutant regions were sequenced in their entirety to verify the absence of additional mutations.

**Full-length PTP-PEST proteins (wild-type and mutant proteins, containing either Asp-199→Ala or Cys-231→Ser mutations) and the wild-type PTP-PEST catalytic domain (amino acids 1 to 305) were expressed in Sf9 cells with recombinant baculovirus (BaculoGold; Pharmingen, San Diego, Calif.) and purified as described in reference 15. Truncated forms of wild-type and mutant PTP-PEST proteins, comprising amino acid residues 1 to 305 of PTP-PEST, were also expressed in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins after subcloning of PTP-PEST DNA in frame downstream of GST in pGEX vectors (Pharmacia Biotech, Inc., Uppsala, Sweden). *E. coli* cells (25 ml) transformed with the appropriate vector were grown to log phase (optical density at 600 nm of approximately 0.5). Fusion protein expression was then induced by addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside, and the cells were grown for 2 to 4 h at 30°C. Cells were harvested by centrifugation, incubated with 50 µg of lysozyme per ml in 3 ml of buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 µg of leupeptin per ml, 5 µg of aprotinin per ml, 0.1% Triton X-100, and 150 mM NaCl, and then lysed by sonication (three times for 10 s each). After removal of insoluble material by centrifugation (20 min at 300,000 × g), fusion proteins were isolated by incubation for 30 min at 4°C with 100 µl of glutathione-Sepharose beads (Pharmacia Biotech, Inc.). The beads were then collected by centrifugation and washed three times with buffer A (20 mM Tris-HCl, [pH 7.4], 1 mM EDTA, 1 mM benzamidine, 1 µg of leupeptin per ml, 1 µg of aprotinin per ml, 10% glycerol, 1% Triton X-100, 100 mM NaCl). This procedure yielded essentially homogeneous fusion protein at a concentration of 1 mg of protein per ml of glutathione-Sepharose beads. PTP1B proteins (wild-type and mutant forms) comprising amino acids 1 to 321 were expressed in *E. coli* cells and purified to homogeneity as described in reference 4.**

**Cell culture, transfection, preparation of lysates, and fractionation.** HeLa (ATCC CCL 2) and COS1 (ATCC CRL 1650) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum; WI38 (ATCC CCL 75), C2C12 (ATCC CRL 1772), and MvLu (ATCC CCL 64) cells were grown in DMEM containing 10% fetal bovine serum; 293 (ATCC CRL 1573) cells were grown in DMEM containing 10% calf serum; and MCF10A (ATCC CRL 10317) cells were grown in 50% DMEM-50% Ham's F-12 medium containing 5% horse serum, 20 ng of epidermal growth factor per ml, 10 µg of insulin per ml, 0.5 µg of hydrocortisone per ml, and 0.25 µg of amphotericin B (Fungizone) per ml. All media also contained penicillin and streptomycin at 100 U/ml and 100 µg/ml, respectively, and all cells were grown at 37°C.

**Calcium phosphate-mediated transfection** was used to introduce cDNA-encoding wild-type and mutant PTP-PEST proteins into COS cells. These were encoded by PTP-PEST cDNA subcloned into the plasmid PMT2, from which expression is driven by an adenovirus major late promoter; 20 µg of DNA was used for transfection of each 10-cm-diameter plate of cells. The level of expression of PTP-PEST constructs was similar in all cases.

Prior to cell lysis, 70 to 90% confluent cultures of cells were treated for 30 min with 0.1 mM pervanadate (20 µl of a fresh solution containing 50 mM sodium metavanadate [NaVO<sub>3</sub>] and 50 mM H<sub>2</sub>O<sub>2</sub> was added to 10 ml of medium).

Treatment of cells with H<sub>2</sub>O<sub>2</sub> and vanadate leads to a synergistic increase in phosphotyrosine levels, presumably due to inhibition of intracellular PTPs by vanadate. The synergism between H<sub>2</sub>O<sub>2</sub> and vanadate has previously been suggested to result from improved accumulation of the resultant oxidized vanadate (pervanadate) within the cells compared with vanadate itself (19). Pervanadate treatment resulted in the appearance of at least 50 prominent phosphotyrosine protein bands in all cell types, whereas untreated cells contained virtually undetectable levels of phosphotyrosine (data not shown).

Cells were lysed in buffer A containing 5 mM iodoacetic acid, which was included in order to inhibit irreversibly cellular PTPs. After incubation at 4°C for 30 min, 10 mM dithiothreitol was added to inactivate any unreacted iodoacetic acid. Insoluble material was then removed by centrifugation for 20 min at 300,000 × g. The resultant lysates were stable with regard to their phosphotyrosine content during long-term (several months) storage at -70°C and during prolonged (at least 20 h) incubation at 4°C in the absence of exogenously added PTPs.

Pervanadate-treated HeLa cell lysate was fractionated by anion-exchange chromatography with a Mono Q fast-performance liquid chromatography (FPLC) column (Pharmacia). The sample (50 mg of total protein at 3 mg/ml in buffer A) was diluted in 3 volumes of buffer B (20 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM benzamidine, 1 µg of leupeptin per ml, 1 µg of aprotinin per ml, 0.1% Triton X-100) prior to loading. Proteins were eluted at a flow rate of 1 ml/min with a linear gradient of 0 to 0.5 M NaCl in buffer B over 20 fractions (1-ml fraction volume), followed by a second gradient of 0.5 to 1.0 M NaCl in buffer B over 5 fractions. Phosphotyrosine-containing proteins were detected within fractions 7 to 21 according to antiphosphotyrosine immunoblotting.

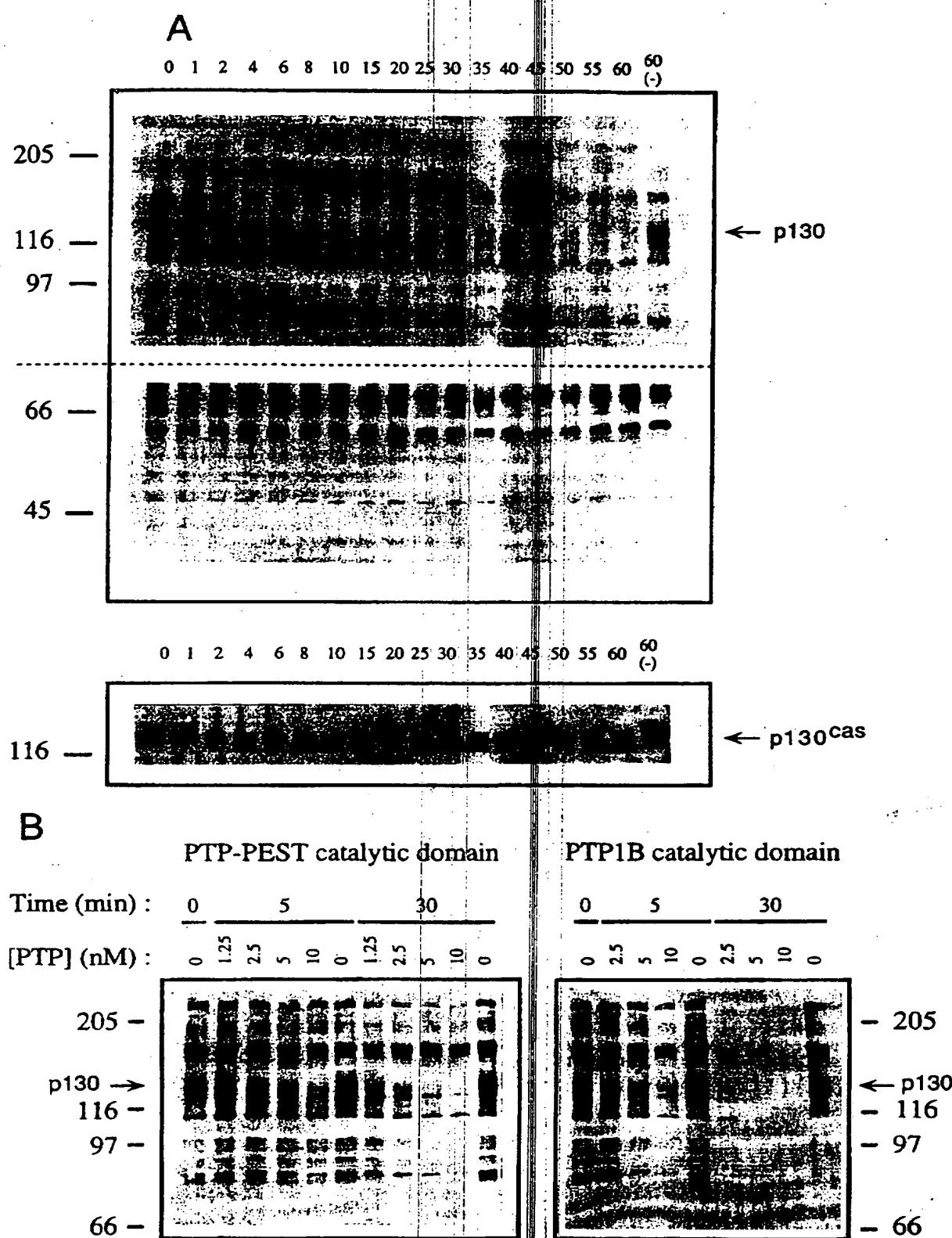
**Dephosphorylation reactions.** Lysates of pervanadate-treated HeLa cells (1 to 2 mg of protein per ml) containing tyrosine-phosphorylated proteins were incubated on ice in the absence or presence of purified active PTPs. Dephosphorylation was terminated by the removal of aliquots (30 µg of protein) into sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and the extent of dephosphorylation was determined by immunoblotting with the monoclonal antibody G104. Assays of PTP activity with tyrosine-phosphorylated <sup>32</sup>P-labelled RCM-lysozyme as the substrate were performed as described in reference 12.

**Antibodies and Immunoblotting.** The PTP-PEST monoclonal antibody AG25 was raised against baculovirus-expressed purified full-length PTP-PEST. The antiphosphotyrosine monoclonal antibody G104 was generated with, as the antigen, phosphotyrosine, alanine, and glycine in a 1:1:1 ratio polymerized in the presence of keyhole limpet hemocyanin with 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide, a method originally described in reference 22. p130<sup>cas</sup> monoclonal antibody was from Transduction Laboratories (Lexington, Ky.); p130<sup>cas</sup> polyclonal antibody was provided by Amy Bouton (University of Virginia). Monoclonal antibody FG6 against PTP1B was provided by David Hill (Calbiochem Oncogene Research Products, Cambridge, Mass.). Visualization of proteins by immunoblotting was achieved by enhanced chemiluminescence with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Life Science, Inc., Arlington Heights, Ill.) and the SuperSignal CL-HRP substrate system (Pierce, Rockford, Ill.).

**Immunoprecipitation and substrate trapping.** Immunoprecipitation of PTP-PEST from transfected COS cells was performed after covalent coupling of monoclonal antibody AG25 to protein A-Sepharose beads (Pharmacia Biotech, Inc.) with the chemical cross-linking agent dimethyl pimelimidate (48). Antibody was first bound to protein A-Sepharose at a concentration of 1 mg/ml of bead volume, and unbound material was then removed by three washes with 0.2 M sodium borate (pH 9). Covalent coupling was achieved by incubation at room temperature for 30 min in the presence of 20 mM dimethyl pimelimidate in 0.2 M sodium borate (pH 9). The beads were then incubated for 1 h with an excess of 0.2 M ethanolamine (pH 8) to block any unreacted cross-linker and washed three times with phosphate-buffered saline prior to storage at 4°C. AG25 beads (10 µl) were used to precipitate transfected PTP-PEST from lysates containing approximately 0.375 mg of protein.

Substrate trapping was performed with various PTP affinity matrices. The full-length PTP-PEST matrix utilized covalent coupled AG25-protein A-Sepharose beads to which purified baculovirus-expressed PTP-PEST protein was bound. Aliquots (10 µl) of AG25 beads were incubated for 2 h at 4°C in 100 µl of buffer A in the presence of 5 µg of purified PTP-PEST (wild-type or mutant forms); unbound PTP-PEST was then removed by three washes with 1 ml of buffer A. The resultant PTP-PEST-AG25-protein A-Sepharose beads contained approximately 2 µg of PTP-PEST per 10-µl aliquot. Substrate trapping was also carried out with glutathione-Sepharose beads bound to bacterially expressed GST fusion proteins containing the catalytic domain of PTP-PEST. PTP1B was also used in substrate-trapping experiments. In this case, the monoclonal antibody FG6 was precoupled to protein A-Sepharose in the absence of cross-linker (2 µg of antibody per 10 µl of beads), and then purified PTP1B proteins were added in excess and incubated at 4°C for 2 h. After removal of unbound PTP1B, 10 µl of beads contained approximately 2 µg of PTP1B.

Pervanadate-treated cell lysates, or column fractions, were used as a source of phosphotyrosine-containing proteins for substrate-trapping experiments. In general, lysates containing 0.25 to 0.5 mg of protein in 0.5 ml of buffer A (including 5 mM iodoacetic acid and 10 mM dithiothreitol) were incubated at 4°C for 2 h in the presence of 10 µl of affinity matrix containing approximately 2 µg of the



**FIG. 1. PTP-PEST selectively dephosphorylates a 130-kDa phosphotyrosine-containing protein from pervanadate-treated HeLa cell lysates.** Aliquots of pervanadate-treated HeLa cell lysates were incubated on ice in the absence [60 (—) lanes] or in the presence of 2 nM purified full-length PTP-PEST (A, upper panel) or the indicated concentrations of PTP-PEST catalytic domain (B, left panel) or PTP1B (37-kDa form) (B, right panel). At the indicated time points (minutes) aliquots containing 30  $\mu$ g of protein were removed into SDS-PAGE sample buffer; phosphotyrosine content was then analyzed by immunoblotting. In panel A, the region below the dashed line was exposed longer (5 min compared with 15 s for the upper portion) in order to display the weak phosphotyrosine bands present in this part of the blot. The lower panel in A is a reprobe of the 130-kDa region of the immunoblot with a rabbit polyclonal antibody to p130<sup>cas</sup>. Sizes are shown in kilodaltons.

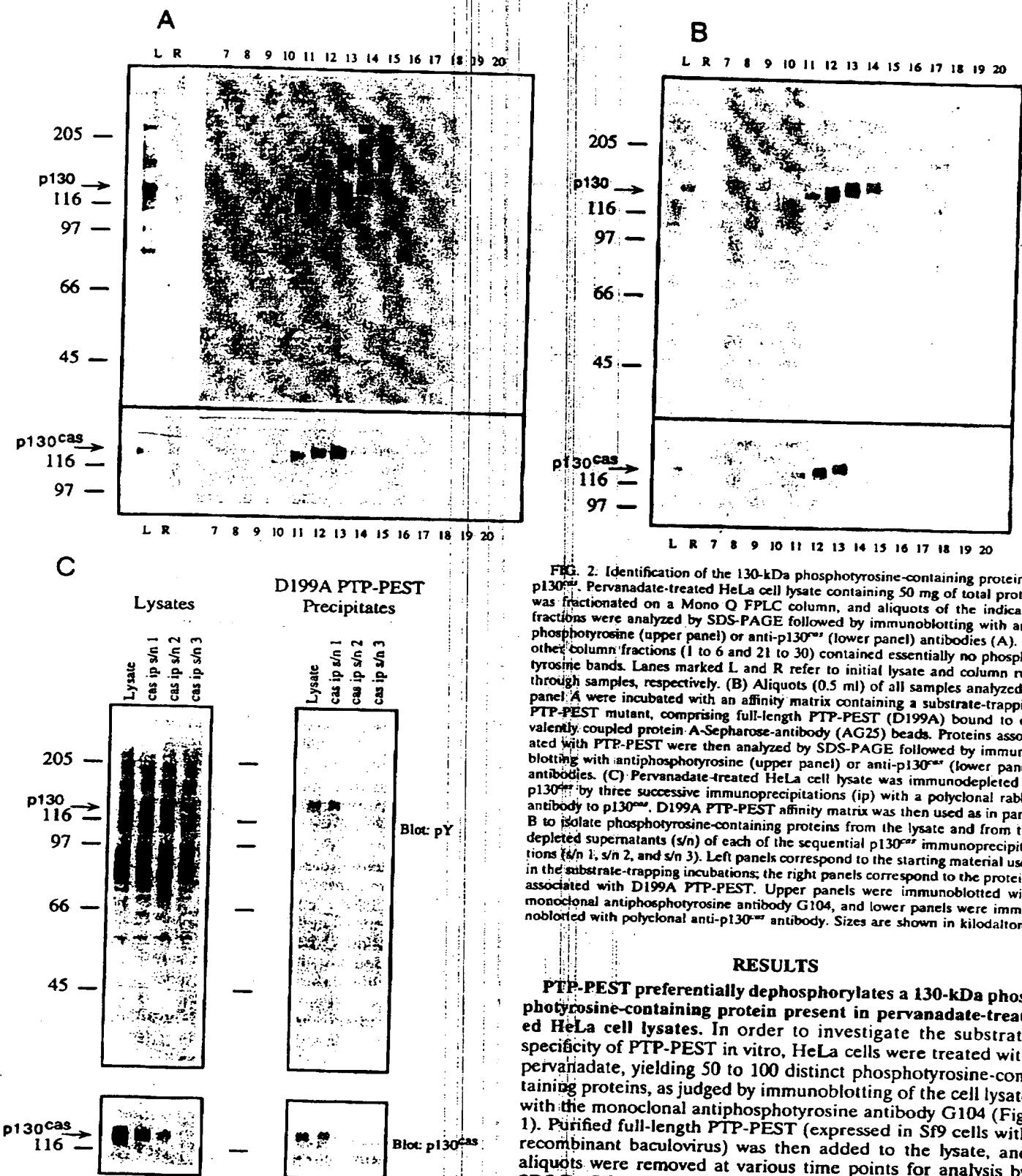


FIG. 2. Identification of the 130-kDa phosphotyrosine-containing protein as p130<sup>cas</sup>. Pervanadate-treated HeLa cell lysate containing 50 mg of total protein was fractionated on a Mono Q FPLC column, and aliquots of the indicated fractions were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine (upper panel) or anti-p130<sup>cas</sup> (lower panel) antibodies (A). All other column fractions (1 to 6 and 21 to 30) contained essentially no phosphotyrosine bands. Lanes marked L and R refer to initial lysate and column run-through samples, respectively. (B) Aliquots (0.5 ml) of all samples analyzed in panel A were incubated with an affinity matrix containing a substrate-trapping PTP-PEST mutant, comprising full-length PTP-PEST (D199A) bound to covalently coupled protein A-Sepharose antibody (AG25) beads. Proteins associated with PTP-PEST were then analyzed by SDS-PAGE followed by immunoblotting with antiphosphotyrosine (upper panel) or anti-p130<sup>cas</sup> (lower panel) antibodies. (C) Pervanadate-treated HeLa cell lysate was immunodepleted of p130<sup>cas</sup> by three successive immunoprecipitations (ip) with a polyclonal rabbit antibody to p130<sup>cas</sup>. D199A PTP-PEST affinity matrix was then used as in panel B to isolate phosphotyrosine-containing proteins from the lysate and from the depleted supernatants (s/n) of each of the sequential p130<sup>cas</sup> immunoprecipitations (s/n 1, s/n 2, and s/n 3). Left panels correspond to the starting material used in the substrate-trapping incubations; the right panels correspond to the proteins associated with D199A PTP-PEST. Upper panels were immunoblotted with monoclonal antiphosphotyrosine antibody G104, and lower panels were immunoblotted with polyclonal anti-p130<sup>cas</sup> antibody. Sizes are shown in kilodaltons.

## RESULTS

PTP-PEST preferentially dephosphorylates a 130-kDa phosphotyrosine-containing protein present in pervanadate-treated HeLa cell lysates. In order to investigate the substrate specificity of PTP-PEST in vitro, HeLa cells were treated with pervanadate, yielding 50 to 100 distinct phosphotyrosine-containing proteins, as judged by immunoblotting of the cell lysate with the monoclonal antiphosphotyrosine antibody G104 (Fig. 1). Purified full-length PTP-PEST (expressed in Sf9 cells with recombinant baculovirus) was then added to the lysate, and aliquots were removed at various time points for analysis by SDS-PAGE followed by antiphosphotyrosine immunoblotting. Surprisingly, a prominent 130-kDa phosphotyrosine protein (p130) was selectively dephosphorylated by PTP-PEST within 10 min, whereas the intensity of the other bands was essentially unchanged even after 60 min of incubation with PTP-PEST (Fig. 1A). Long incubations with higher concentrations of PTP-PEST (greater than 100-fold) resulted in the complete

appropriate PTP protein. Unbound proteins were then removed from the samples by three washes with 1 ml of buffer A, and bound material was collected by addition of 50  $\mu$ l of SDS-PAGE sample buffer followed by heating at 95°C for 5 min; proteins bound to the beads were then analyzed by SDS-PAGE followed by immunoblotting.

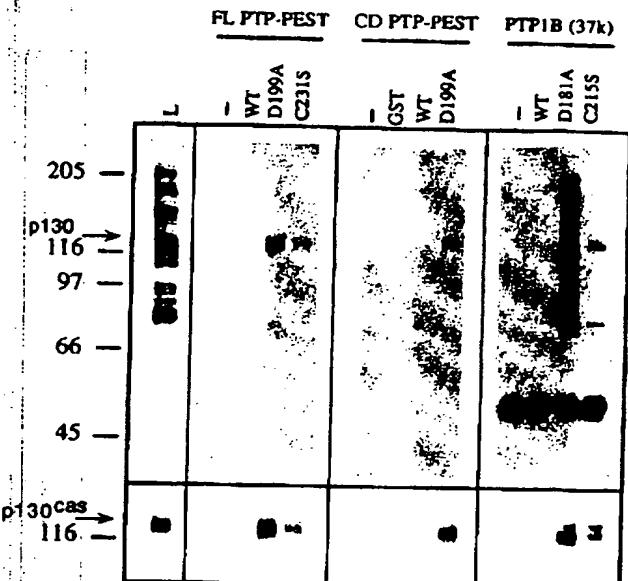
removal of all phosphotyrosine bands from the lysate. However, under all conditions tested, p130 was found to be dephosphorylated more rapidly than all other proteins present.

The selective dephosphorylation of p130 by PTP-PEST was also observed with a truncated form of the phosphatase (amino acid residues 1 to 305) which essentially contains only the catalytic domain of the enzyme (Fig. 1B, left panel). This result suggests that the striking substrate preference displayed by PTP-PEST in this analysis is an inherent property of the phosphatase catalytic domain, whereas the C-terminal 500 amino acid residues have little discernible effect on the substrate specificity of the enzyme.

The specificity of the interaction between PTP-PEST and p130 was addressed with the catalytic domain of PTP1B (amino acid residues 1 to 321) in dephosphorylation reactions. When added at molar concentrations similar to those used for the catalytic domain of PTP-PEST, PTP1B was found to dephosphorylate fully most of the phosphotyrosine-containing proteins present in the pervanadate-treated HeLa lysate (Fig. 1B). In addition, the time course of dephosphorylation of p130 was not significantly more rapid than that of the other phosphotyrosine proteins dephosphorylated by PTP1B. PTP-PEST and PTP1B display similar specific activities (40,000 to 50,000 U/mg of protein) when assayed in vitro (4, 15); the ability of PTP1B to dephosphorylate all tyrosine-phosphorylated proteins present under conditions in which PTP-PEST exclusively dephosphorylates p130<sup>cas</sup> (Fig. 1B, 30-min incubations at 5 to 10 nM PTP) therefore suggests that the substrate specificity of PTP-PEST is relatively stringent and that p130 is a potential physiologically significant substrate for PTP-PEST.

**Identification of the phosphotyrosine-containing p130 protein as p130<sup>cas</sup> by substrate trapping with an inactive mutant form of PTP-PEST.** Phosphotyrosine-containing proteins from a pervanadate-treated HeLa cell lysate were fractionated by anion-exchange chromatography with a Mono Q FPLC column. Antiphosphotyrosine immunoblotting of the resultant column fractions showed that the p130 phosphotyrosine protein eluted as a single peak in fractions 11 to 14 (approximately 0.3 M NaCl) (Fig. 2A, upper panel). In view of the abundance of tyrosine-phosphorylated p130 in HeLa lysates, it appeared likely that p130 represents a previously identified phosphotyrosine-containing 130-kDa protein. Several potential candidates were identified in the literature, including the focal adhesion kinase p125<sup>FAK</sup>, Ras-GAP, gp130, and p130<sup>cas</sup>. Of these candidates, p130<sup>cas</sup> has been identified as a particularly prominent phosphotyrosyl protein in a wide variety of systems, including v-crk (30, 32) and src (23, 42)-transformed fibroblasts, integrin-mediated cell adhesion (35, 37, 57), and cells stimulated by a variety of mitogenic agents (27, 41, 43, 50, 61). Therefore we tested the possibility that the p130 phosphotyrosine protein corresponds to p130<sup>cas</sup> by immunoblotting the Mono Q fractions with an antibody to p130<sup>cas</sup>. The 130-kDa band corresponding to p130<sup>cas</sup> eluted in the same fractions as the p130 tyrosine-phosphorylated protein and displayed a similar apparent molecular mass (Fig. 2A, lower panel), suggesting that they might represent the same protein. Furthermore, p130<sup>cas</sup> immunoprecipitated from these fractions was found to be phosphorylated on tyrosyl residues (data not shown).

A mutant form of PTP-PEST (D199A) was generated by site-directed mutagenesis; the mutant enzyme was then purified after expression with recombinant baculovirus. When assayed with tyrosine-phosphorylated RCM-lysozyme as a substrate, the purified mutant enzyme exhibited a specific activity which was approximately 10,000-fold lower than that of the wild-type enzyme (16). This purified protein was bound to an affinity matrix composed of an anti-PTP-PEST monoclonal



**FIG. 3. Specificity of the interaction between inactive mutant PTP-PEST and tyrosine-phosphorylated p130<sup>cas</sup>.** Various affinity matrices were incubated with 0.3 mg of pervanadate-treated HeLa cell lysate, and proteins associated with the beads were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine (upper panel) or anti-p130<sup>cas</sup> (lower panel) antibodies. FL PTP-PEST matrix consists of full-length PTP-PEST proteins bound to covalently coupled protein A-Sepharose antibody (AG25) beads; CD PTP-PEST matrix consists of glutathione-Sepharose beads with GST fusion proteins containing amino acids 1 to 305 of PTP-PEST; PTP1B (37k) matrix consists of amino acids 1 to 321 of PTP1B coupled to protein A-Sepharose antibody (FG6) beads. Control samples (—) consisted of matrix alone (antibody-protein A-Sepharose or glutathione-Sepharose beads); GST sample contained glutathione transferase protein; WT refers to wild-type PTP proteins; other samples contained the indicated point mutations. The lane marked L consists of 30  $\mu$ g of lysate protein. The prominent band at 50 kDa in the PTP1B lanes corresponds to the antibody heavy chain. Sizes are shown in kilodaltons.

antibody (AG25) covalently coupled to protein A-Sepharose beads and was then incubated with each of the Mono Q fractions. After 45 min of incubation, proteins associating with the mutant PTP-PEST were collected by centrifugation, the beads were washed, and SDS-PAGE sample buffer was added. Associated proteins were then analyzed by immunoblotting with the monoclonal antiphosphotyrosine antibody G104. The mutant PTP-PEST protein was found to associate with a single phosphotyrosine-containing protein, the molecular mass (130 kDa) and Mono Q elution position (fractions 11 to 14) of which coincided with those of p130<sup>cas</sup> (Fig. 2B, upper panel). Immunoblotting of the PTP-PEST-associated proteins with the p130<sup>cas</sup> antibody demonstrated that the 130-kDa tyrosine-phosphorylated protein trapped by the mutant PTP-PEST is indeed p130<sup>cas</sup> (Fig. 2B, lower panel). These data further support the notion that p130<sup>cas</sup> is a potential physiologically relevant substrate for PTP-PEST.

In order to confirm that p130<sup>cas</sup> alone is recognized by PTP-PEST, p130<sup>cas</sup> protein was specifically depleted from the lysates by immunoprecipitation. When these samples were subsequently incubated with mutant PTP-PEST, none of the remaining tyrosine-phosphorylated proteins associated with the mutant enzyme (Fig. 2C), indicating that the 130-kDa tyrosine-phosphorylated protein observed in mutant PTP-PEST precipitates consists solely of p130<sup>cas</sup>.

Having identified the tyrosine-phosphorylated protein associated with the mutant form of PTP-PEST as p130<sup>cas</sup>, the antiphosphotyrosine immunoblot from Fig. 1A was reprobed

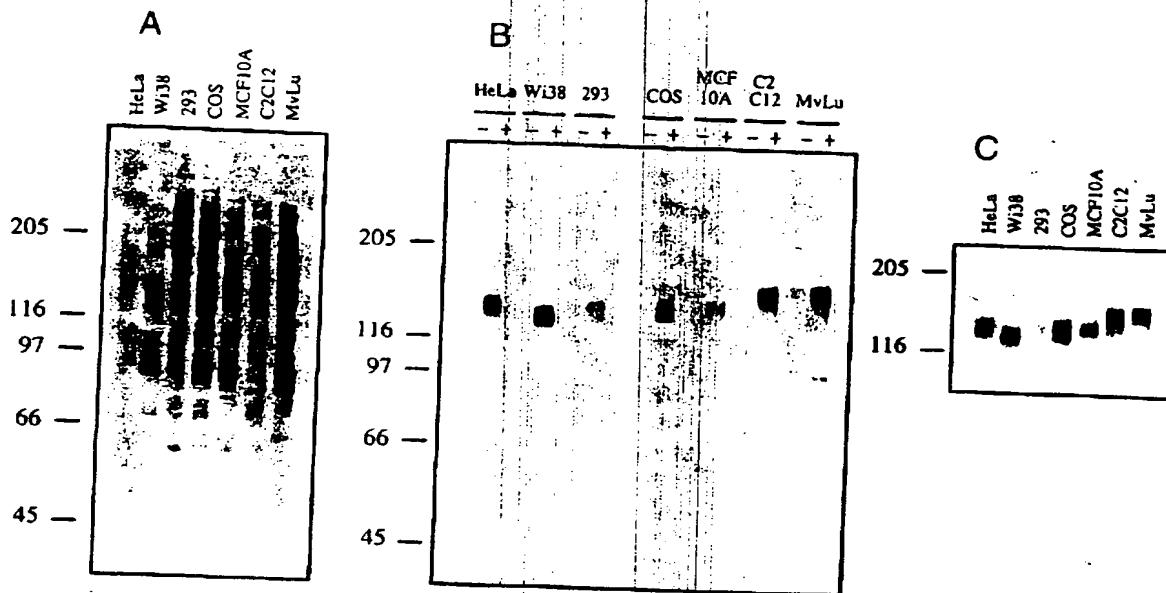


FIG. 4. Interaction of inactive mutant PTP-PEST with tyrosine-phosphorylated  $p130^{\text{cas}}$  from different cell lines. The indicated cell lines were treated with pervanadate, and the resultant lysates (30  $\mu\text{g}$  of total protein) were analyzed by SDS-PAGE followed by antiphosphotyrosine immunoblotting (A). Aliquots (0.3 mg) were incubated with the PTP-PEST (D199A) affinity matrix (+) or control matrix (-); the associated proteins were then analyzed by SDS-PAGE and immunoblotting with antiphosphotyrosine (B) or anti- $p130^{\text{cas}}$  (C) antibodies. In panel B, the left portion of the blot (HeLa, WI38, and 293 samples) was exposed for 5 min, whereas the remainder was exposed for 30 s.

with a  $p130^{\text{cas}}$  polyclonal antibody (Fig. 1A, lower panel). The band corresponding to  $p130^{\text{cas}}$  migrated at the same molecular mass as the 130-kDa phosphotyrosine-containing protein which was rapidly dephosphorylated by PTP-PEST. Furthermore,  $p130^{\text{cas}}$  displayed increased electrophoretic mobility within 10 min of incubation with PTP-PEST, indicating that  $p130^{\text{cas}}$  was rapidly dephosphorylated by PTP-PEST under these conditions. In addition, these data confirm that the observed reduction in antiphosphotyrosine antibody staining (Fig. 1A, upper panel) was due to the dephosphorylation of  $p130^{\text{cas}}$  by PTP-PEST and was not due to proteolysis during the incubation.

**Determination of structural features of PTP-PEST involved in the specific interaction with tyrosine-phosphorylated  $p130^{\text{cas}}$ .** The interaction between  $p130^{\text{cas}}$  and PTP-PEST was investigated further in substrate-trapping experiments using various purified mutant forms of PTP-PEST to precipitate proteins from pervanadate-treated HeLa lysates. The wild-type full-length phosphatase was found to be incapable of stable association with tyrosine-phosphorylated  $p130^{\text{cas}}$ , whereas both the PTP-PEST (D199A) mutant protein and a mutant lacking the active site cysteine residue (C231S) specifically precipitated  $p130^{\text{cas}}$  from the lysate (Fig. 3). The inability of the wild-type phosphatase to precipitate tyrosine-phosphorylated  $p130^{\text{cas}}$  presumably reflects the transient nature of the normal interaction between PTP-PEST and tyrosine-phosphorylated  $p130^{\text{cas}}$ , which is likely to be concluded as soon as  $p130^{\text{cas}}$  is dephosphorylated by PTP-PEST.

Since the C-terminal 500-amino-acid segment of PTP-PEST contains several proline-rich regions which resemble Src homology 3 (SH3) domain binding sequences, it appeared plausible that the specificity of the interaction between PTP-PEST and  $p130^{\text{cas}}$  might depend to some extent on association of these segments with the SH3 domain of  $p130^{\text{cas}}$ . The possible contribution of the C-terminal segment of PTP-PEST in the observed specific interaction of PTP-PEST with  $p130^{\text{cas}}$  was therefore addressed in further substrate-trapping experiments

with GST fusion proteins containing the catalytic domain of PTP-PEST alone in both wild-type and mutant (D199A) forms. The mutant catalytic domain of PTP-PEST fused to GST was found to precipitate phosphotyrosyl  $p130^{\text{cas}}$  specifically, whereas both the wild-type fusion protein and GST alone failed to precipitate tyrosine-phosphorylated  $p130^{\text{cas}}$  (Fig. 3). The specific interaction between PTP-PEST and  $p130^{\text{cas}}$  observed in these experiments therefore appears to be an intrinsic property of the catalytic domain of PTP-PEST, emulating the observed preference of the active PTP-PEST catalytic domain for dephosphorylation of  $p130^{\text{cas}}$  in vitro (Fig. 1B).

**Specificity of the interaction between mutant PTP-PEST and tyrosine-phosphorylated  $p130^{\text{cas}}$ .** In view of the relative abundance of tyrosine-phosphorylated  $p130^{\text{cas}}$  in the pervanadate-treated HeLa cell lysate (Fig. 1), we considered the possibility that the observed selective binding of PTP-PEST substrate-trapping mutant proteins to  $p130^{\text{cas}}$  was substrate directed (reflecting the abundance of this potential substrate relative to the other phosphotyrosine-containing proteins present in the lysate) rather than enzyme directed (reflecting a genuine substrate preference of PTP-PEST); this possibility was addressed in two ways. First, inactive mutant forms of the catalytic domain of PTP1B were used to trap potential substrates for this enzyme from the pervanadate-treated HeLa lysates. Again, we found that the wild-type phosphatase was incapable of stable interaction with any phosphotyrosine-containing protein, whereas mutant variants of the PTP1B phosphatase domain (comprising Cys or Asp mutations analogous to those described above for PTP-PEST) associated with many tyrosine-phosphorylated proteins (Fig. 3). This was especially apparent for the aspartic acid mutant of PTP1B (D181A), which appeared to precipitate essentially all phosphotyrosine-containing proteins from the lysate with similar efficacy (compare Fig. 3, lysate and PTP1B D181A lanes). The C215S mutant of PTP1B displayed lower affinity for phosphotyrosine-containing proteins than the D181A mutant; however, longer exposure of the immunoblot in Fig. 3 demonstrated that the

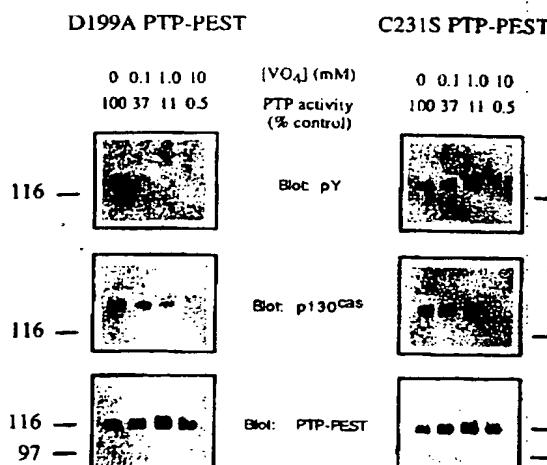


FIG. 5. Vanadate inhibition of the interaction between mutant PTP-PEST and tyrosine-phosphorylated p130<sup>cas</sup>. PTP-PEST affinity matrix, comprising full-length PTP-PEST (D199A or C231S) bound to covalently coupled protein A-Sepharose antibody (AG25) beads, was incubated for 10 min on ice in the presence of the indicated concentrations of sodium orthovanadate (VO<sub>3</sub>). The samples were then incubated with aliquots (0.3 mg) of pervanadate-treated HeLa cell lysate; associated proteins were then analyzed by SDS-PAGE and immunoblotting with antiphosphotyrosine (upper panel), anti-p130<sup>cas</sup> (middle panel), or anti-PTP-PEST (lower panel) antibodies. The activity of wild-type PTP-PEST was also determined under the same conditions, with tyrosine-phosphorylated <sup>32</sup>P-labelled RCM-lysozyme as a substrate.

C215S mutant of PTP1B was still capable of forming stable complexes with the majority of the tyrosine-phosphorylated proteins present (data not shown). These data emphasize the specific nature of the interaction between PTP-PEST and p130<sup>cas</sup>, which appears to be a property peculiar to the PTP-PEST catalytic domain rather than a feature shared by all PTP catalytic domains. Nevertheless, it is important to note that *in vivo*, the subcellular distribution of the full-length form of PTP1B is restricted by targeting to the cytoplasmic face of membranes of the endoplasmic reticulum (14). This subcellular targeting imposes constraints on substrate recognition by PTP1B so that only selected substrates are recognized *in vivo* (13).

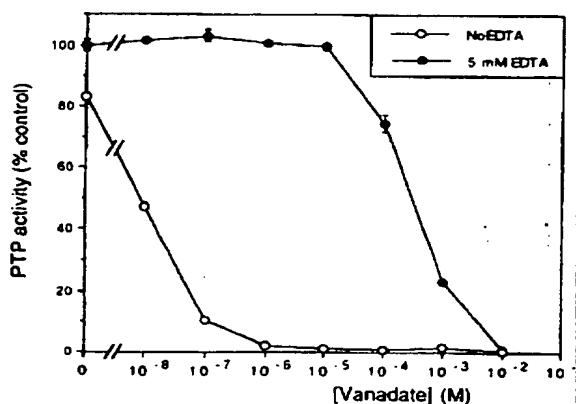


FIG. 6. Effect of EDTA on the inhibition of PTP-PEST by vanadate. Full-length wild-type PTP-PEST was incubated for 5 min with the indicated concentrations of sodium orthovanadate in the absence (○) or presence (●) of 5 mM EDTA. PTP activity against tyrosine-phosphorylated <sup>32</sup>P-labelled RCM-lysozyme was then assayed in the presence of the same combinations of inhibitor and EDTA. All results are expressed relative to the activity of PTP-PEST in the absence of vanadate and in the presence of 5 mM EDTA.

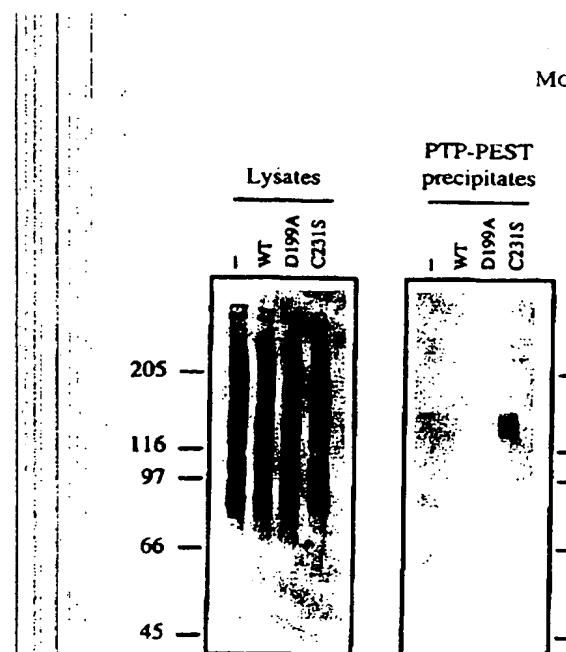


FIG. 7. Association of endogenous p130<sup>cas</sup> with transfected PTP-PEST in COS cells. cDNAs encoding wild-type (WT) or mutant full-length PTP-PEST or the vector alone (—) were transfected into COS cells, which were treated with pervanadate for 30 min prior to lysis. Aliquots (30 μg of protein) of each lysate were immunoblotted with antiphosphotyrosine antibody (left panel). Lysates (50 μg of protein) were also incubated with covalently coupled protein A-Sepharose-anti-PTP-PEST (AG25) beads, and associated proteins were analyzed by SDS-PAGE and immunoblotting with antiphosphotyrosine antibody (right panel).

The specificity of the interaction between PTP-PEST and p130<sup>cas</sup> was addressed further after pervanadate treatment of several different cell lines (Wi38, 293, COS, MCF10A, C2C12, and MvLu), yielding a different array of tyrosine-phosphorylated proteins in each case (Fig. 4A). The lysates were then incubated in the presence of mutant PTP-PEST (D199A), and tyrosine-phosphorylated proteins associating with PTP-PEST were analyzed as described in the legend to Fig. 3. In each case, the D199A mutant PTP-PEST protein precipitated a single broad phosphotyrosine band with an apparent molecular weight of between 120,000 and 150,000 in different cell lines, whereas the affinity matrix alone failed to precipitate any phosphotyrosine-containing protein (Fig. 4B). Immunoblotting of the precipitates with a p130<sup>cas</sup> antibody revealed that the protein precipitated from all cell lysates corresponded to p130<sup>cas</sup> (Fig. 4C); the observed molecular weight variation between different cell lines presumably reflects either species differences in the molecular weight of p130<sup>cas</sup>, expression of different alternatively spliced forms (45), or different levels of tyrosine phosphorylation of p130<sup>cas</sup>. The relative abundance of tyrosine-phosphorylated p130<sup>cas</sup> in the PTP-PEST precipitates appeared to correlate approximately with the abundance of p130<sup>cas</sup> protein in the lysates (data not shown). Surprisingly, regardless of the abundance of tyrosine-phosphorylated p130<sup>cas</sup> in the lysates, p130<sup>cas</sup> was invariably the only phosphotyrosine-containing protein in the precipitates—even in 293 cell lysates, which contain very little p130<sup>cas</sup> protein but which display a wide variety of other abundantly tyrosine-phosphorylated proteins (Fig. 4A, lane 3). Similarly, when lysates of pervanadate-treated 293 cells (containing tyrosine-phosphorylated p130<sup>cas</sup> in amounts which are undetectable by antiphosphotyrosine immunoblotting of the lysate) were incubated with active PTP-PEST (as described in the legend to Fig. 1), no visible dephosphorylation of any phosphotyrosine band occurred (16). These results indicate that the affinity of PTP-PEST for p130<sup>cas</sup> is substantially greater than for any other substrate present and

further emphasize the remarkable substrate selectivity of PTP-PEST for p130<sup>cas</sup> observed in these experiments.

**Association of tyrosine-phosphorylated p130<sup>cas</sup> with inactive mutant PTP-PEST is blocked by vanadate.** We have consistently observed that, in contrast to the inactive mutant PTP-PEST, the wild-type enzyme fails to associate in a stable complex with tyrosine-phosphorylated p130<sup>cas</sup> (Fig. 3), suggesting that the observed association is active site directed. In order to investigate this possibility, mutant PTP-PEST (D199A or C231S) was incubated with the PTP inhibitor vanadate at various concentrations prior to addition of p130<sup>cas</sup> in p130<sup>cas</sup> lysate. The extent of association of p130<sup>cas</sup> with PTP-PEST was then analyzed as described in the legend to Fig. 3. The association of p130<sup>cas</sup> with D199A PTP-PEST was found to be potently disrupted by vanadate, with a concentration dependence similar to that of vanadate inhibition of wild-type PTP-PEST (Fig. 5, left panels). Surprisingly, C231S PTP-PEST was unaffected in its ability to associate with p130<sup>cas</sup> at vanadate concentrations (0.1 to 1 mM) which significantly disrupted the association with D199A PTP-PEST, although 10 mM vanadate effectively blocked the interaction with C231S PTP-PEST (Fig. 5, right panels). These data support a model for PTP inhibition by vanadate in which potent enzyme inhibition arises through direct interaction of vanadate ions with the thiolate anion of the PTP active site cysteine residue; in the absence of the cysteine residue, vanadate is therefore a much less potent inhibitor of enzyme-substrate binding. This model is supported by recently reported structural data, which indicated the presence of a covalent bond between vanadate and the active site cysteine residue of the PTP Yop51 (10). Our data therefore support the notion that the stable association of mutant PTP-PEST with tyrosine-phosphorylated p130<sup>cas</sup> is mediated by direct interactions between active site residues within PTP-PEST, in particular the active site cysteine residue, and phosphotyrosine moieties within p130<sup>cas</sup>.

The requirement for millimolar concentrations of vanadate to inhibit both wild-type PTP-PEST activity and the interaction of p130<sup>cas</sup> with D199A PTP-PEST was somewhat surprising, since previous reports have indicated that PTP inhibition occurs at considerably lower vanadate concentrations (58, 62). This result appears to be a consequence of the presence of EDTA in both lysis and assay buffers, which we have found to affect considerably the 50% inhibitory concentration of PTP-PEST inhibition by vanadate (Fig. 6). Thus, in the absence of EDTA, the 50% inhibitory concentration of vanadate is approximately 10 nM, whereas inclusion of 5 mM EDTA in the assay buffer increases the 50% inhibitory concentration to approximately 0.3 mM (Fig. 6). This effect appears to result from chelation of vanadate by EDTA, since intermediate EDTA concentrations have less effect on the dose-response curve of PTP-PEST inhibition by vanadate (data not shown). This effect of EDTA probably accounts for the ability of mutant PTP-PEST to interact potently with p130<sup>cas</sup> in lysates of p130<sup>cas</sup>-treated cells prepared in 1 mM EDTA (Fig. 3).

**Association of endogenous p130<sup>cas</sup> with transfected mutant PTP-PEST in COS cells.** The experiments described above strongly suggest that p130<sup>cas</sup> represents a potential physiologically significant substrate for PTP-PEST. In order to assess whether PTP-PEST interacts with p130<sup>cas</sup> in intact cells, COS cells were transfected with plasmids encoding wild-type or mutant forms of PTP-PEST (D199A or C215S). The cells were treated with p130<sup>cas</sup> 30 min prior to lysis, PTP-PEST proteins were immunoprecipitated, and associated tyrosine-phosphorylated proteins were analyzed by antiphosphotyrosine immunoblotting of the resultant precipitates. Under these conditions, the phosphotyrosine-containing band corresponding to

p130<sup>cas</sup> was again unique in its ability to associate with the C231S PTP-PEST protein (Fig. 7), indicating that p130<sup>cas</sup> can be specifically selected by PTP-PEST as a substrate in an intracellular context in the presence of a large number of alternative possible substrates. Neither the wild-type nor the D199A form of PTP-PEST was capable of a stable interaction with tyrosine-phosphorylated p130<sup>cas</sup> in p130<sup>cas</sup>-treated COS cells (Fig. 7). The binding of both wild-type and D199A PTP-PEST to tyrosine-phosphorylated p130<sup>cas</sup> under these conditions is most likely prohibited by the presence of p130<sup>cas</sup> bound to the active site cysteine residue of PTP-PEST (Fig. 5 and reference 10), which effectively excludes the binding of phosphotyrosine residues of p130<sup>cas</sup>. The ability of the C231S mutant PTP-PEST to associate in a stable complex with p130<sup>cas</sup> in the presence of p130<sup>cas</sup> again indicates that this mutant protein is largely unaffected by p130<sup>cas</sup>. As an alternative to p130<sup>cas</sup> treatment, COS cells cotransfected with v-src and either wild-type or mutant PTP-PEST proteins were used as a source of tyrosine-phosphorylated proteins. Although the level of tyrosine phosphorylation observed in these cells was much lower than that in p130<sup>cas</sup>-treated cells, a specific interaction between mutant PTP-PEST and tyrosine-phosphorylated p130<sup>cas</sup> was again observed (data not shown).

In summary, these observations lend further support to the suggestion that the exclusive interaction between PTP-PEST and tyrosine-phosphorylated p130<sup>cas</sup>, which we have consistently observed, is entirely active site directed and therefore reflects the genuine, inherent highly restricted substrate preference of PTP-PEST for p130<sup>cas</sup>.

## DISCUSSION

The results described in this paper implicate p130<sup>cas</sup> as a physiologically relevant substrate for PTP-PEST. Furthermore, the observed stringency and exclusivity of the interaction between PTP-PEST and p130<sup>cas</sup> in a wide variety of cell lines suggest that p130<sup>cas</sup> may be a unique high-affinity substrate for PTP-PEST, although the possibility that other significant PTP-PEST substrates exist cannot be excluded at present. In particular, it is unclear whether p130<sup>cas</sup>-treated cells display a complete spectrum of all possible tyrosine-phosphorylated proteins; in fact, this appears unlikely, since p130<sup>cas</sup> treatment presumably results only in an increase in tyrosine phosphorylation of proteins which are to some extent constitutively phosphorylated, but which are normally rapidly dephosphorylated within the cell. Potential substrates lacking from p130<sup>cas</sup>-treated cells therefore presumably include substrates of protein tyrosine kinases which are normally present in an inactive state, such as ligand-stimulated receptor protein tyrosine kinases and the recently described calcium-regulated kinase PYK2 (28). Regardless of these considerations, the ability of PTP-PEST to select p130<sup>cas</sup> exclusively as a substrate from lysates of several different cell lines, containing a combined total of at least 50 different potential substrates (many of which presumably contain multiple sites of phosphorylation), clearly demonstrates that the substrate specificity of PTP-PEST is highly restricted.

Many intracellular PTPs are limited in their substrate availability because of strict confinement within a particular subcellular location; examples include PTP1B, which is localized to the cytoplasmic face of the endoplasmic reticulum (14), and TCPTP, which is either nuclear (55) or localized to the endoplasmic reticulum, depending upon which alternative spliced form is expressed (29). Alternatively, certain PTPs appear to be highly regulated, requiring activation before appreciable

activity can be demonstrated. For example, the SH2 domain-containing PTPs, SHP1 and SHP2, display relatively little activity in vitro, but can be considerably activated by several mechanisms, including C-terminal truncation (65), addition of certain phospholipids (66), or SH2 domain-mediated binding of appropriate phosphotyrosine-containing peptides (26). However, PTP-PEST exhibits high specific activity in vitro (35,000 U/mg) and is a predominantly (90 to 95%) soluble PTP within cells (16); in principle, therefore, it may act potently on any substrate accessible to the cytoplasm. This accessibility may partly underlie the necessity for PTP-PEST to possess an inherently constrained substrate specificity. The demonstration that mutant PTP-PEST is capable of exclusively associating with p130<sup>cas</sup> in an intracellular context (Fig. 6) in the presence of many other tyrosine-phosphorylated proteins is an indication that the narrow substrate specificity of the enzyme may result in PTP-PEST having a negligible influence on the phosphorylation state of the majority of tyrosine-phosphorylated proteins within the cell, even though those substrates are largely accessible to PTP-PEST.

The nature of the physiologically relevant tyrosine phosphorylation sites on p130<sup>cas</sup> is unknown. The larger splice variant of p130<sup>cas</sup> contains 31 tyrosine residues; 16 of these have the sequence YxxP (45), which resembles the preferred binding site for the SH2 domain of the adapter protein Crk (52), and it appears likely that a subset of these motifs within p130<sup>cas</sup> represent in vivo phosphorylation sites (31, 43, 45, 46). p130<sup>cas</sup> was initially identified as a prominent tyrosine-phosphorylated protein in cells transformed by the viral oncogene *v-crk* (30, 32) and by activated variants of *src* (23, 42). In both cases, tyrosine-phosphorylated p130<sup>cas</sup> is associated with Src and Crk proteins (24, 32, 42, 45), and these interactions appear to involve largely SH2 domain-mediated binding of these proteins to phosphotyrosine residues within p130<sup>cas</sup> (24, 34). Optimal binding of Src to p130<sup>cas</sup> also requires an intact Src SH3 domain (24), which is thought to bind a proline-rich sequence (RPLPSPP) in p130<sup>cas</sup> (34). The role of p130<sup>cas</sup> in cellular transformation by the *v-crk* and *v-src* oncogenes is unclear, although there is a general correlation between the level of tyrosine phosphorylation of p130<sup>cas</sup> and the degree of transformation in cells expressing different forms of Crk or Src (24, 31). Furthermore, enhanced tyrosine phosphorylation of p130<sup>cas</sup> has also been observed in cells transformed by c-Ha-ras and by ornithine decarboxylase overexpression (1); expression of antisense cDNA encoding p130<sup>cas</sup> in these cells results in a partial reversion of the transformed phenotype (1). These observations suggest that aberrant tyrosine phosphorylation of p130<sup>cas</sup> is a common feature of cells transformed by several disparate mechanisms and that p130<sup>cas</sup> may be required for full manifestation of the transformed state. Dephosphorylation of p130<sup>cas</sup> by PTP-PEST is therefore a potentially important regulatory mechanism for counteracting the transforming effects of various oncogenes.

Tyrosine phosphorylation of p130<sup>cas</sup> has been observed in fibroblasts during integrin-mediated cell adhesion to extracellular matrix proteins (35, 37, 57). Under these conditions, with an antibody (4F4) that predominantly recognizes tyrosine-phosphorylated p130<sup>cas</sup> (24, 37), it was shown that phosphorylated p130<sup>cas</sup> is localized to focal adhesions (37), whereas fractionation studies have demonstrated that the normal cellular location of the majority of nonphosphorylated p130<sup>cas</sup> is the cytosol (45). Furthermore, in *crk*-transformed fibroblasts, tyrosine-phosphorylated p130<sup>cas</sup> is detected only in insoluble fractions (45), suggesting that both cell adhesion-mediated phosphorylation and transformation-mediated phosphorylation of p130<sup>cas</sup> are associated with redistribution of the protein

from the cytosol to focal adhesions. The precise mechanism of this redistribution is unclear, but it was recently reported that the SH3 domain of p130<sup>cas</sup> is capable of interacting with a proline-rich region in the focal adhesion kinase, FAK (40). It is therefore plausible that the redistribution of tyrosine-phosphorylated p130<sup>cas</sup> may be driven by its association with FAK, which is constitutively associated with focal adhesions due to its C-terminal focal adhesion targeting domain (20, 47). The sequestration of tyrosine-phosphorylated p130<sup>cas</sup> in focal adhesions both in transformed cells and after integrin-mediated cell adhesion strongly suggests a role for p130<sup>cas</sup> in signalling events in this region of the cell. One consequence of the redistribution of tyrosine-phosphorylated p130<sup>cas</sup> is likely to be that in addition to localizing p130<sup>cas</sup> to a region of the cell containing abundant protein tyrosine kinase activity, the phosphorylated protein will be relatively inaccessible to the cytosolic phosphatase PTP-PEST. This raises the possibility that the role of PTP-PEST in dephosphorylating p130<sup>cas</sup> may be to prevent inappropriate tyrosine phosphorylation of the cytosolic pool of p130<sup>cas</sup>, thus preventing formation of signalling complexes assembled around tyrosine-phosphorylated p130<sup>cas</sup> in inappropriate cellular locations. In addition, a rapid dephosphorylation of p130<sup>cas</sup> has been observed during cell disaggregation induced by disruption of the interaction between LFA-1 integrin receptors and ICAM-1 ligand molecules (38). This observation supports a role for PTPs in regulating integrin-mediated cell adhesion processes through the dephosphorylation of p130<sup>cas</sup>.

Previous studies have utilized active site cysteine mutant variants of PTPs in order to investigate the substrate specificity or substrate binding mechanism of these enzymes (18, 21, 33, 51, 53). Our data indicate that mutation of the invariant catalytically essential aspartic acid residue (analogous to D199 in PTP-PEST) generally results in PTPs with improved substrate-trapping properties compared with active site cysteine mutants (Fig. 3). There are two features of the D199A mutant that may contribute to its ability to trap substrates more efficiently than the cysteine mutant. First, during the catalytic process, a thiol-phosphate intermediate is generated in which the active site cysteine residue forms a covalent bond with the phosphate of the substrate phosphotyrosine moiety (17). Normally at this point in the reaction, hydrolysis of the bond between the phosphate moiety and the substrate tyrosine residue occurs, a reaction which is catalyzed by the invariant aspartic acid residue. In the absence of this aspartic acid residue, the lytic part of the reaction occurs much more slowly; the substrate molecule is therefore trapped by the direct interaction with the active site cysteine. In addition, the substrate molecule is held by hydrophobic interactions arising from the movement of a loop in the enzyme structure (residues 179 to 187 in PTP1B and 197 to 205 in PTP-PEST, within which the catalytically essential aspartic acid residue normally resides) which partially closes the active site cleft of the enzyme (21). The second feature of the aspartic acid mutant protein which facilitates high-affinity interactions with substrate molecules is the resultant loss of negative charge on this loop of the protein. This modification is likely to result in reduced electrostatic repulsion of the loop from the negatively charged phosphate moiety of the substrate, allowing more complete closure of the active site cleft and strengthening the hydrophobic interactions between the enzyme and the tyrosine moiety of the substrate phosphotyrosine.

The identification of p130<sup>cas</sup> as a preferred substrate for PTP-PEST supports the idea that some members of the PTP family of enzymes may have specific physiological roles in dephosphorylation of particular tyrosine-phosphorylated substrates. The methods outlined in this paper are generally applicable to

any PTP and should therefore prove useful in delineating the substrate preference of other PTP family members. In particular, the use of mutant, catalytically impaired PTPs to trap, and thereby isolate, potential substrates will greatly facilitate the identification of physiologically important substrates for individual PTPs, leading to improved understanding of the roles of these enzymes in regulation of cellular processes.

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